

Demonstration of Coinfection with and Recombination by Caprine Arthritis-Encephalitis Virus and Maedi-Visna Virus in Naturally Infected Goats[▽]

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Recombination of different strains and subtypes is a hallmark of lentivirus infections, particularly for human immunodeficiency virus, and contributes significantly to viral diversity and evolution both within individual hosts and within populations. Recombinant viruses are generated in individuals coinfecting or superinfected with more than one lentiviral strain or subtype. This, however, has never been described in vivo for the prototype lentivirus maedi-visna virus of sheep and its closely related caprine counterpart, the caprine arthritis-encephalitis virus. Cross-species infections occur in animals living under natural conditions, which suggests that dual infections with small-ruminant lentiviruses (SRLVs) are possible. In this paper we describe the first documented case of coinfection and viral recombination in two naturally infected goats. DNA fragments encompassing a variable region of the envelope glycoprotein were obtained from these two animals by end-limiting dilution PCR of peripheral blood mononuclear cells or infected cocultures. Genetic analyses, including nucleotide sequencing and heteroduplex mobility assays, showed that these goats harbored two distinct populations of SRLVs. Phylogenetic analysis permitted us to assign these sequences to the maedi-visna virus group (SRLV group A) or the caprine arthritis-encephalitis virus group (SRLV group B). SimPlot analysis showed clear evidence of A/B recombination within the *env* gene segment of a virus detected in one of the two goats. This case provides conclusive evidence that coinfection by different strains of SRLVs of groups A and B can indeed occur and that these viruses actually recombine in vivo.

Lentiviruses display a remarkable variability due to their error-prone reverse transcriptase and, as a result of their diploid genomes, recombine frequently and efficiently during replication. Indeed, the role of recombination of different strains and subtypes is now recognized as a major factor in the generation of diversity in the human immunodeficiency virus (HIV) pandemic (36). Dual infections are the prerequisite for such recombination events and can be divided into coinfections and superinfections.

Several reports to date have documented mixed infection in an individual, including HIV type 1 (HIV-1)/HIV-2 dual infections (11), double infection with two different subtypes of HIV-1 (3, 14), and mixed infection with multiple strains of HIV-1 subtype B (7, 39).

The experimental infection of a goat with both the caprine arthritis-encephalitis virus (CAEV) and the maedi-visna virus (MVV) has been described (13). However, mixed infections with these two closely related small-ruminant lentiviruses (SRLVs) occurring under field conditions have to date not been reported. CAEV and MVV infect goats and sheep (21–23) and cause persistent infection for life, inducing slowly debilitating disease syndromes in a fraction of the infected animals. Several molecular epidemiological studies have shown that CAEV and MVV are related, albeit genetically distinct, viruses that fall

into two major phylogenetic clusters (29, 31, 38). The genetic diversity of SRLVs is extensive, and phylogenetic analysis (31) of the nucleotide sequence of the *gag-pol* region has led to the definition of four new groups: group A corresponds to the heterogeneous MVV type and can be further subdivided into seven distinct subtypes, designated A1 to A7; group B refers to the genetically less complex CAEV type and comprises only two distinct lineages, called subtypes B1 and B2; and two additional SRLV groups (C and D) have recently been identified on the basis of their great genetic divergence with the two previous groups. Interspecies transmission occurs in the field, as suggested by the detection of some SRLV subtypes in both sheep and goats (16, 28, 29, 38). Recently, direct evidence was found proving that, in mixed flocks of sheep and goats, the SRLV subtypes A4 (32) and B1 (26) do indeed jump the species barrier. In view of these results, we reasoned that double infection may also occur in SRLV-infected goats and sheep. To test this hypothesis, we selected goats from a commercial flock of about 300 animals that had a seroprevalence of 100% based on their serological reactivity to a panel of synthetic peptides encompassing a variable region (SU5) of the envelope glycoprotein of SRLV (5). The reactivity pattern to these SU5 peptides provides information on the type of virus infecting the animals, which permitted us to differentiate between CAEV- and MVV-infected animals (19). We selected two goats that reacted strongly to SU5 peptides of both CAEV and MVV origin and postulated that these animals may be coinfecting with the two virus types. The results obtained provide the first direct evidence that dual infections occur in small ruminants and that the infecting viruses recombine in vivo,

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generating new viruses with chimerical CAEV-MVV envelope glycoproteins. Recombined viruses may have important implications for the evolution and epidemiology of SRLVs in goat and sheep populations.

MATERIALS AND METHODS

Animals. This study was conducted with goats from a commercial dairy flock of about 300 animals of different breeds (Saanen and Alpine). No SRLV control measures were implemented in this herd.

Synthetic peptides. Synthetic peptides were designed according to SU5 sequences obtained from GenBank as described previously (19). The peptides were synthesized and purified by Primm (Milan, Italy).

Serological analysis. All blood samples were collected by jugular venipuncture. Sera were separated from the blood clots and tested with MVV/CAEV enzyme-linked immunosorbent assays (ELISAs) (Institut Pourquier, Montpellier, France) for seroprevalence determination. SU5 ELISAs were performed to obtain information on the phylogenetic origin of the infecting viruses. These tests were performed as previously described in detail (19). Briefly, SU5 synthetic peptides were dissolved in a carbonate-bicarbonate buffer (0.1 M sodium carbonate [pH 9.6]) at 5 µg/ml and allowed to adsorb to ELISA plates (Maxisorp immunoplate; Nunc, Roskilde, Denmark) overnight at 4°C (100 µl per well). After several washes, residual adsorption sites were saturated with phosphate-buffered saline–Tween 20 containing 5% fat-free milk. Sera were tested at a 1:25 dilution. After being washed, the plates were incubated with peroxidase-conjugated protein G. Bound conjugate was visualized with a hydrogen peroxide–2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS) solution and the optical density (OD) measured at a 405-nm wavelength after 30 min.

PBMC isolation. Blood samples from goats 12 and 13 were collected 2 months prior to and 12 to 24 h after delivery. One hundred milliliters of blood was collected in 2 mM EDTA Vacutainers. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation through Ficoll-Paque Plus (GE Healthcare Europe GmbH, Milan, Italy) at 800 × g for 30 min at 10°C. Pellets of 5 × 10⁶ cells were prepared for DNA and RNA extraction and stored at –80°C. The remaining PBMCs were washed twice in Hanks' solution with 2 mg of EDTA/ml (600 × g for 10 min at 15°C) for virus isolation.

PBMC cultures and virus isolation. Freshly isolated PBMCs were resuspended in macrophage differentiation medium consisting of RPMI 1640 with glutamine (2 mM), HEPES buffer (10 mM), 2-mercaptoethanol (0.05 mM), gentamicin (0.05 mg/ml), and heat-inactivated newborn lamb serum (20%; Invitrogen, Carlsbad, CA) and cultured in a 14-ml Falcon polypropylene round-bottom tube (Becton Dickinson, Milan, Italy) at 37°C in 5% CO₂. After 7 days of culture, a twofold dilution of 20 × 10⁶ PBMCs was seeded in duplicate in Costar six-well plates containing semiconfluent goat synovial membrane (GSM) cells as indicator cells, grown in minimal essential medium (MEM) supplemented with 5% fetal calf serum, and prepared as follows. GSM cells, originally derived from the explanted carpal synovial membrane of a colostrum-deprived newborn goat (21), were resuspended in 10 ml of MEM–Earle medium supplemented with 10% fetal bovine serum, seeded at a density of 10⁵ cells/well, and incubated at 37°C in 5% CO₂ in Costar six-well plates (Corning BV Life Sciences, Schiphol-Rijk, The Netherlands). Nonadherent cells were removed after 24 h, and fresh medium was added. Every second day, the cells were washed to eliminate proliferating lymphocytes. After 1 week, the GSM cells were split and transferred into Costar 24-well plates. Thereafter, the cell cultures were passaged weekly for at least 5 weeks. Cytopathic effects (CPEs) indicative of virus replication such as the presence of syncytia or cell lysis were monitored upon fixing the cells with formalin (10%) and staining them with May–Grünwald–Giemsa solutions. When the maximum CPE was observed, cells and clarified culture supernatants were harvested and stored at –80°C for DNA and RNA extraction.

Nucleic acid extraction, sequence amplification, and cloning. DNA from PBMCs and GSM cell-PBMC cocultures was extracted from pellets containing ≤ 5 × 10⁶ cells using commercial silica gel spin columns selective for genomic DNA (QIAamp DNA blood mini kit; QIAGEN, Milan, Italy) in accordance with the manufacturer's instructions.

RNA was extracted from GSM-PBMCs and from clear culture supernatants with TRIzol reagent (Invitrogen, Carlsbad, CA) and was retrotranscribed using SuperScript III RNase H-free reverse transcriptase (RT; Invitrogen, Carlsbad, CA).

Initially, DNA was subjected to nested PCR to amplify a 520-bp fragment of the *pol* gene, as described previously (26). Subsequent PCR amplifications, however, were restricted to the *env* region using Platinum *Taq* DNA polymerase high fidelity (Invitrogen, Carlsbad, CA).

To avoid template resampling (17), DNA from PBMCs and DNA and cDNA from cocultured cells and supernatants were subjected to limiting-dilution semi-nested PCR to amplify a 607-bp fragment encompassing the C-terminal part of surface (SU4-SU5) and the N-terminal part of transmembrane (TM) proteins encoded by the *env* gene (bp 7482 to 8089 of CAEV-CO [accession number M33677]) with the following primers: 563, 564, and 567 (19).

To avoid potential PCR contamination artifacts, extractions of nucleic acid, preparation of PCR mixes, and analyses of the amplified products were performed in separate rooms. Additionally, samples were never processed in parallel but individually. Multiple SRLV-negative controls (no-template controls and negative controls, including DNA extracted from uninfected goats) were run in parallel for each reaction.

PCR products of approximately 610 bp from five or more independent PCRs, obtained by end-limiting dilutions of the input DNA for each goat, were cloned with the TOPO TA cloning kit (pCR 4-TOPO Vector; Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Plasmid DNAs were prepared by following the rapid extraction method of Birnboim and Doly (6) and digested with EcoRI restriction enzyme (Invitrogen, Carlsbad, CA) to confirm the size of the insert.

HMA. The genotypes of SRLVs in blood and coculture proviral and viral samples were examined using the heteroduplex mobility assay (HMA) developed by Delwart et al. (8). Twenty-five of the resulting insert-containing plasmids from each subject were used to generate clonal PCR products using second-round PCR primers. Pairwise HMAs were then used to identify plasmids containing divergent sequence variants through the generation of slowly migrating DNA heteroduplexes. Briefly, heteroduplexes were obtained by mixing equal amounts of a plasmid-derived PCR product to be characterized with a reference PCR-amplified DNA sample from the CAEV-CO strain in the presence of an annealing buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.8], 2 mM EDTA). To study intrahost variability, PCR products obtained from PBMC and coculture viral and proviral sample amplifications were subjected to HMAs with the reference CAEV-CO strain in order to provide a baseline heteroduplex pattern because of quasispecies diversity and coinfection in vivo. Denaturation and renaturation of DNA were carried out by heating the mixtures at 97°C for 5 min, followed by cooling in ice. The heteroduplexes that formed were then subjected to electrophoresis on a nondenaturing 8% polyacrylamide gel in 1× Tris-borate-EDTA buffer at 150 V for about 2 h 30 min and stained with ethidium bromide. A 100-bp ladder (Invitrogen, Carlsbad, CA) was used as a molecular weight marker. The mobility of the heteroduplexes formed between sufficiently divergent molecules is reduced relative to that of the fully complementary homoduplex molecules. Plasmids containing distinct *env* variants as well as multiple plasmids showing no apparent difference by HMA were then sequenced.

Sequencing and sequence analysis. To confirm the qualitative results obtained by HMA, DNA from 20 to 30 clones was purified by using the Wizard plus minipreps DNA purification system (Promega, Milano, Italy) and sequenced by CRIBI Services (CRIBI, Padova, Italy) on an ABI377 sequencer by using the ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase (Perkin-Elmer, Applied Biosystems).

Sequences from each cloned viral DNA sample were aligned by the Clustal W multiple sequence alignment program (35), and pairwise genetic distances were calculated with the MEGA version 2.1 software (15) according to the Tamura-Nei substitution model and by applying the default setting, with the exception that all sites with gaps were ignored. Phylogeny construction was carried out using the neighbor-joining method (30) implemented in the MEGA software with the Tamura-Nei gamma distance (34). The statistical confidence of the topologies was assessed with 1,000 bootstrap replicates (9). Finally, plots of nucleotide similarity between SRLV isolates were created with the SimPlot software (version 1.6; <http://sray.med.som.jhmi.edu/SCSoftware/simplot/>) to define genomic regions that display significant percentages of nucleotide sequence identity and provide indications of possible recombination events.

Nucleotide sequence accession numbers. All new nucleotide sequences were deposited in the GenBank database and are available under accession numbers EF154461 to EF154466 for recombinant clones, EF154467 to EF154483 for PBMC12 clones, and EF154484 to EF154511 for PBMC13 clones.

RESULTS

The serological SRLV status of 300 goats from a commercial flock was determined using a commercial ELISA, and the seroprevalence was 100%. Thirty goats were selected at random, and, by applying a standard PCR protocol, a 512-kb

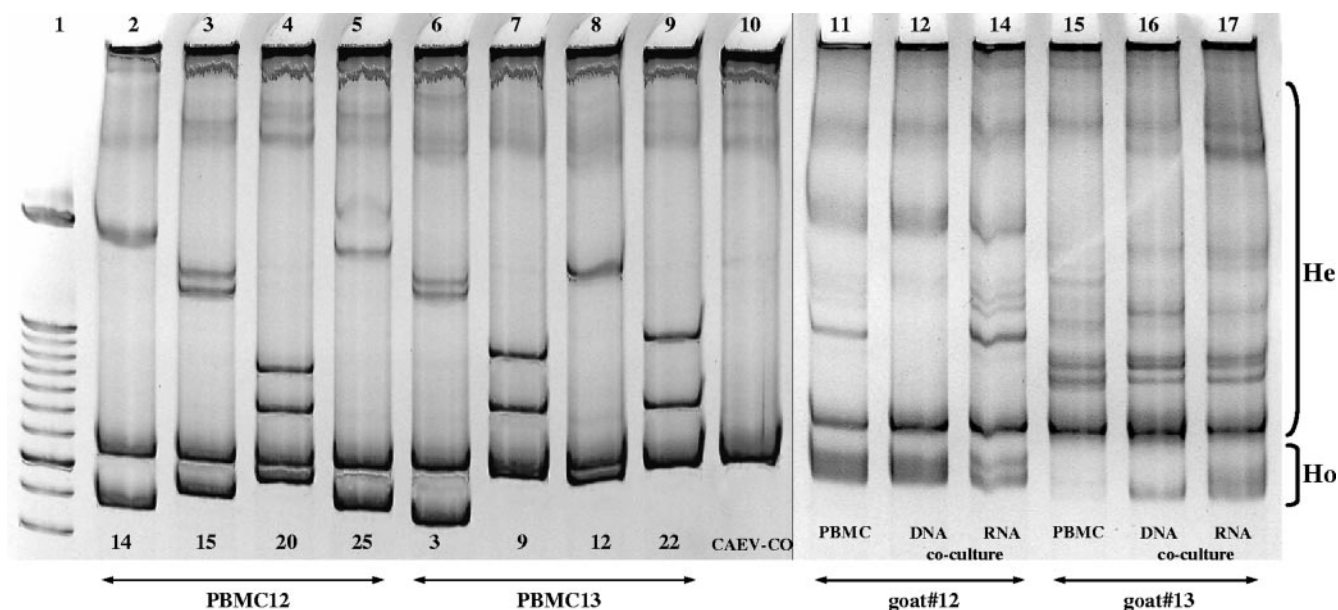


FIG. 1. HMA of a fragment of the SRLV *env* gene. Lane 1, 100-bp DNA ladder; lanes 2 through 9, PCR-amplified fragments spanning the SU4-SU5 and TM regions of the SRLV *env* gene from the PBMCs of goat 12 (clones 14, 15, 20, and 25) and goat 13 (clones 3, 9, 12, and 22) mixed and reannealed with the corresponding fragment of the prototype CAEV-CO strain; lane 10, homoduplex formation after reannealing of the only CAEV-CO strain; lanes 11 through 17, intrahost variability detected with PCR products obtained from PBMC DNA, coculture DNA, and retrotranscribed RNA. HMA analyses were performed with the reference CAEV-CO strain. Heteroduplexes were resolved on an 8% nondenaturing polyacrylamide gel. Ho and He refer to the positions of homoduplex and heteroduplex migrations, respectively. Photographs of the ethidium bromide-stained gels are displayed as reversed pictures.

fragment encompassing a portion of the *pol* gene (the proximal one-third of RT) was amplified from each animal. Sequencing of these PCR products revealed the presence of both SRLV groups A and B in the herd (data not shown).

Two seropositive goats (goat 12 and goat 13) were selected for their strong reactivity to SU5 peptides matching CAEV and MVV Env sequences. Antibodies to SU5 typically show type-specific reactions; therefore, we suspected the presence of a co- or superinfection in these two animals (19).

Cocultures of PBMCs from goat 12 and goat 13 with GSM developed a CPE characterized by the presence of syncytia and cell lysis. Seminested PCR performed with DNA and cDNA from coculture cells and with cDNA from culture supernatants amplified a 607-bp fragment of the *env* region, proving that virus replication took place. Cloning and sequencing of these PCR products confirmed the presence of both viruses in proviral and viral forms.

Seminested PCR products were amplified with *env*-specific primers from PBMC DNA of the two goats and cloned into plasmids. Seventeen to 29 clones were sequenced to determine the variability of the *env* region within each host.

Additionally, the *env* clones were analyzed by HMA and, in accordance with the sequence data, formed slow- or fast-migrating heteroduplexes with the prototype CAEV-CO strain, confirming the presence of different virus strains in these animals. As expected, HMA intrasample analysis of products amplified from PBMC proviral DNA, coculture proviral DNA, and coculture viral RNA tested with the prototype CAEV-CO showed the presence of multiple, slow- and fast-migrating heteroduplexes, confirming HMA as a reliable, fast screening assay for multiple infections. The PBMC- and culture-derived

env PCR products comigrated with similar heteroduplexes on the HMA gels. The heteroduplex patterns were distinctive for every individual analyzed, representing viral sequences unique to each subject. Duplicate limiting-dilution PCRs of each sample were run in parallel to confirm the adequateness of template sampling.

The reproducibility of the HMA pattern obtained indicates that all the major genotypic variants were adequately sampled. Representative HMA profiles are shown in Fig. 1. The presence of the same set of SRLV subtypes in multiple samples from the two goats separated in time (2 months prior to and 12 to 24 h after delivery) and processed independently practically excludes contamination as a potential source for these results (data not shown).

Seventeen *env* clones from goat 12 and 29 from goat 13 were sequenced. Sequence comparison revealed that the mean percentages of divergence among all the sequenced PBMC-derived clones of goat 12 were $15.7\% \pm 1.3\%$ (mean \pm standard deviation; ranging from 0.2 to 36.7%) at the nucleotide level and $14.9\% \pm 1.5\%$ (ranging from 0.3 to 28.2%) at the amino acid level, while in goat 13, the mean sequence variations were $19.3\% \pm 1.4\%$ (ranging from 0 to 40.1%) and $17.4\% \pm 1.7\%$ (ranging from 0 to 28%) at the nucleotide and amino acid levels, respectively. Envelope sequence analysis revealed that PBMC-derived clones from goat 12 (Fig. 2) were characterized by a proviral population divided into two main clusters: cluster I was more related to CAEV-like strains with sequences characterized by a mean nucleotide variability of $0.4\% \pm 0.2\%$, and cluster II was more closely related to MVV-like strains with sequences characterized by a mean nucleotide variability of $1.3\% \pm 0.3\%$. Two clones were quite distinct from the two

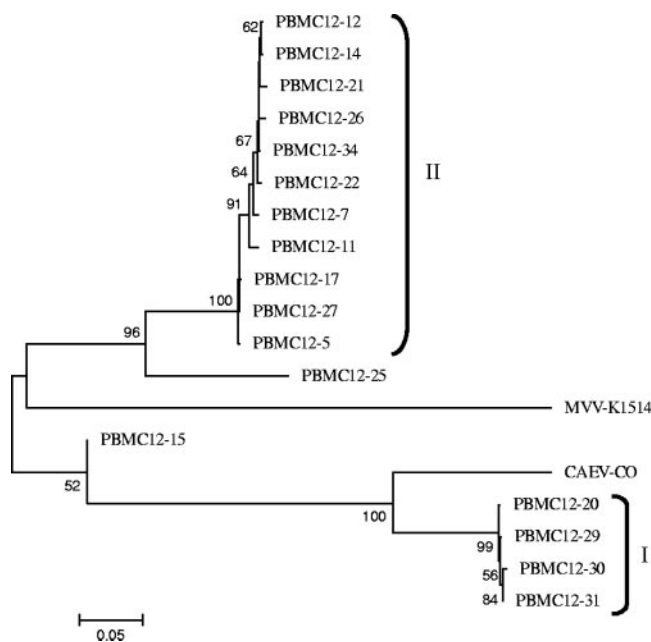


FIG. 2. Phylogenetic analysis of SRLV *env* nucleotide sequences (SU4-SU5 and partial TM regions) from PBMCs of goat 12 and other SRLV subtypes (CAEV-CO prototype for SRLV group B and MVV K1514 prototype for SRLV group A) as constructed by the neighbor-joining method. Clone PBMC12-15 represents the recombinant between SRLV group A and B isolates. The bootstrap values above 50% (1,000 runs) are shown at the nodes. Cluster I corresponds to SRLV group B sequences; cluster II corresponds to MVV-like sequences.

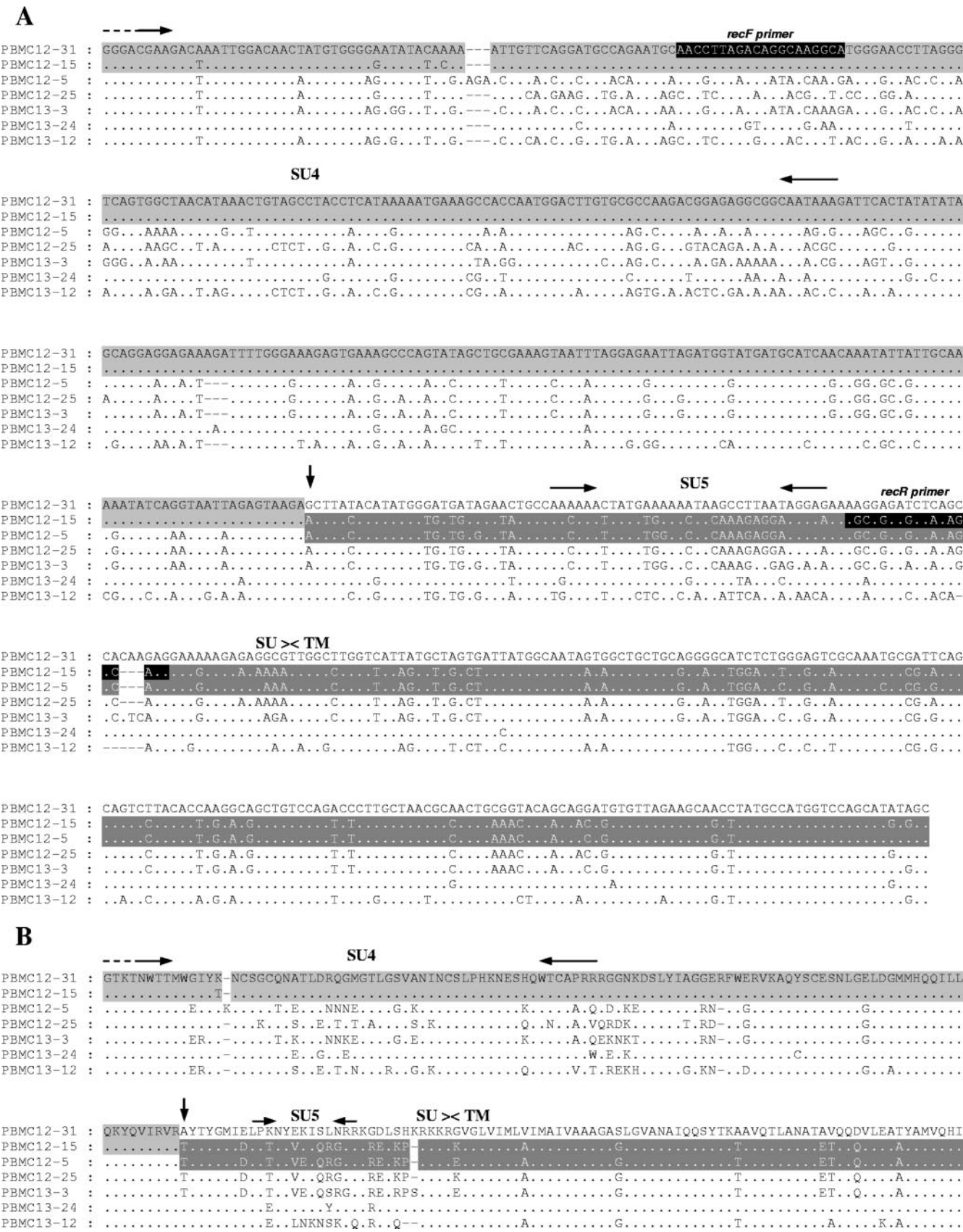
main sequence clusters detected in this goat. Clone PBMC12-25 was separated from cluster II by a large bootstrap value (>80) and was characterized by mean nucleotide divergences of 13.9% from cluster II sequences and 28.2% from cluster I sequences, while clone PBMC12-15 showed mean nucleotide divergences of 14.7% from cluster II sequences and 13.9% from cluster I sequences. This indicates a distant phylogenetic origin of these strains or suggests that a recombination event took place between viruses belonging to the two virus clusters infecting this animal. In order to explore this possibility, we analyzed all sequences with the SimPlot program (version 2.5), an interactive, 32-bit software program that permits us to detect potential recombination events. All the *env* sequences of both animals were aligned separately as a query sequence with all sequences from each animal and with a panel of reference sequences representing SRLV groups A and B. In brief, SimPlot calculates and plots the percent identity of the query sequence relative to a panel of reference sequences in a sliding window, which is moved across the alignment in steps. As expected, for both animals the sequences showed the best match with group A or B, excluding recombination events in the region analyzed. The only exception was the sequence of clone PBMC12-15, which at its 5' end was similar to sequences of the group B viruses but at the 3' end was closer to sequences of group A, with a breaking point at nucleotide position 328. The sequence of clone PBMC12-15, shown in Fig. 2, was derived from an end-limit dilution PCR that contained more than one target provirus. To exclude the possibility that this sequence was the product of an in vitro

PCR- or cloning-recombination artifact (18), we designed primers specific for the 5' and 3' ends of clone PBMC12-15. These primers amplified a specific fragment of 346 bp exclusively from clone PBMC12-15 but not from the other MVV- and CAEV-like clones tested individually or in the form of different MVV-CAEV plasmid mixtures, used as negative controls. DNA from PBMCs or from DNA and cDNA obtained from cocultured cells and their supernatants (obtained from both animals) were subjected to an independent nested PCR using these particular primers in the second round of amplification. As expected, the 346-bp fragment was detected only by testing material obtained from goat 12, such as PBMC DNA, DNA and RNA from cocultured cells, and RNA from the clarified supernatant of these cocultures. No amplification was observed using the DNA and cDNA isolated from the cells and cocultures of goat 13. Additionally, several clones obtained from these independent amplification experiments were sequenced and found to be identical to the originally amplified sequence, except for single-base differences that did not affect the subtype character of the sequence (Fig. 3). This proves that the putative recombinant genome was indeed present in goat 12's proviral DNA from PBMCs and cocultured cells and that this chimerical virus is able to replicate in GSM cell cocultures. The absence of this particular recombinant genome from goat 13 tends to exclude PCR contamination as a source for the results described above.

Phylogenetic analysis of PBMC provirus sequences from goat 13 (Fig. 4) confirmed the formation of two different clusters, as observed for goat 12, with sequences more closely related to CAEV-like strains or MVV-like strains but with a greater intragroup variability. Sequences belonging to cluster I, more closely related to CAEV-like strains, are characterized by a mean nucleotide variability of $1.2\% \pm 0.3\%$, while mean distance within cluster II, a group of sequences related to MVV-like strains, has a $2.9\% \pm 0.5\%$ variability. The PBMC13-12 clone is characterized by a mean nucleotide divergence of 26% from cluster I and of 24.9% from cluster II. The phylogenetic relationship between isolates in cluster II from goat 13 suggests that they represent divergent quasispecies derived from a common ancestral virus.

DISCUSSION

For the first time, to our knowledge, a dual infection of goats with SRLVs belonging to the CAEV and MVV groups was demonstrated under field conditions. Not surprisingly, these dually infected animals were found in a commercial flock, where SRLV control measures had not been implemented, resulting in an SRLV seroprevalence of nearly 100% and frequent detection of animals showing clinical signs of SRLV-induced diseases such as arthritis. The introduction of MVV in this flock could not be traced back to a previous contact with infected sheep but most likely resulted from the purchase of goats infected with MVV-like viruses. Infection of goats with MVV-like viruses has been demonstrated under experimental and field conditions and appears to be quite common in certain regions (4; S. Valas and H.-R. Vogt, personal communication). The main routes of transmission in this herd include direct contact between animals, as the goats are housed in large barns



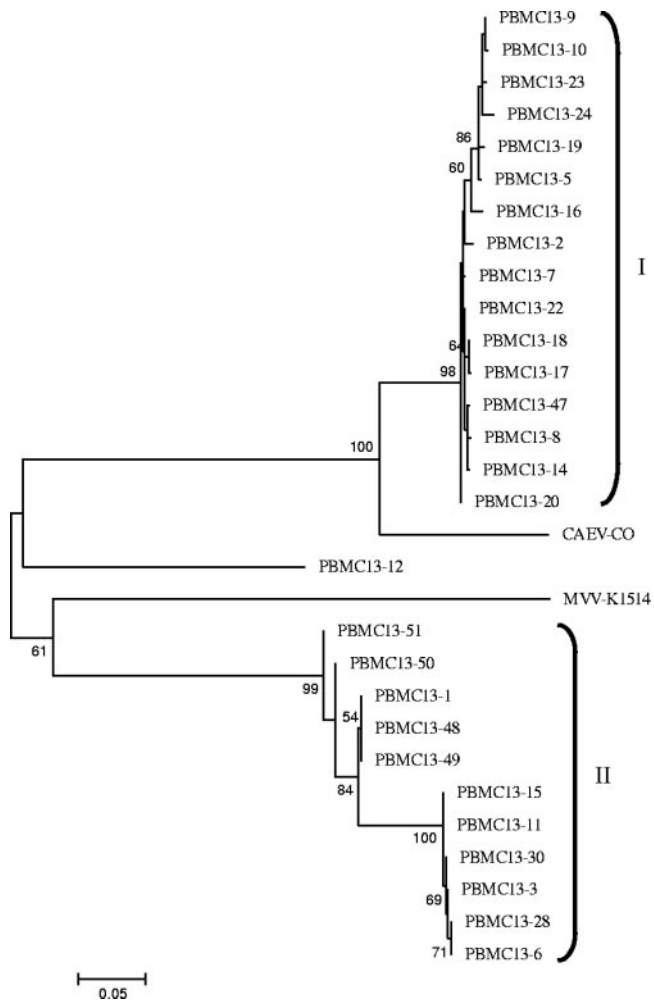


FIG. 4. Phylogenetic analysis of SRLV *env* nucleotide sequences (SU4-SU5 and partial TM regions) from PBMCs of goat 13 and other SRLV subtypes (CAEV-CO prototype for SRLV group B and MVV K1514 prototype for SRLV group A) as constructed by the neighbor-joining method. The bootstrap values above 50% (1,000 runs) are shown at the nodes. Cluster I corresponds to SRLV group B sequences; cluster II corresponds to MVV-like sequences.

sharing a common milking parlor, and, since kids are not separated from dams at birth, colostrum-milk ingestion.

The presence of a dual infection in goats 12 and 13 was initially suspected because of the peculiar serological reactivity of these animals, with SU5 peptides encompassing a highly variable region of the Env glycoprotein (5, 37). We have previously demonstrated that antibody binding to these peptides is by and large type specific, and the strong reactivity of these

sera with both CAEV- and MVV-derived SU5 peptides suggested the presence of a dual infection. PCR amplification and sequence analysis using primers specific for the *pol* region of SRLV corroborated this assumption (data not shown).

Ovine and caprine lentiviruses differ in their cytopathic phenotypes (27), which may reflect their *env* sequence diversities. The prototypic Icelandic MVV strains, such as 1514, induce syncytia and lysis of infected tissue culture monolayers and are classified as lytic. In contrast, CAEV strains, as illustrated by the prototypic CAEV-CO strain, induce syncytia with persistent infection of tissue culture monolayers and are classified as persistent or nonlytic. As shown by Jolly and Narayan (13), GSM cells can sustain the simultaneous replication of CAEV and MVV. Cocultures of PBMCs isolated from the two goats with GSM cells showed both types of CPE, confirming that these viruses were able to replicate in culture and adding a new piece of evidence to the hypothesis that these animals had been infected with viruses that had both MVV- and CAEV-like characteristics. PCR and RT-PCR results confirmed the presence of replicating viruses in these GSM cocultures.

Amplification and sequencing of a variable region of Env encompassing the SU4 and SU5 domains of SRLV permitted a precise analysis of the sequences' distribution in these animals and proved the concomitant infection of these two goats with viruses belonging to both SRLV group A (MVV) and group B (CAEV). Phylogenetic analysis confirmed the presence in both animals of two clearly distinct viral quasiespecies in which the MVV-like sequences, belonging to cluster II, are characterized by a greater variability than is found in the CAEV-like sequences. This variability may reflect the faster evolution of MVV in the caprine host.

Previous works have estimated the evolution rates of SRLVs in animals infected over the long term (1, 12). In light of these results, the large genetic distance observed between the sequence of clone PBMC12-25 in goat 12 and that of clone PBMC13-12 in goat 13 from both cluster I (28.2 and 26%) and cluster II (13.9 and 24.9%) sequences could hardly be attributed to the evolution of these viruses within their hosts. Furthermore, the great genetic distance (23.9%) between the two clones PBMC12-25 and PBMC13-12 suggests that these are two different strains; therefore, we hypothesized that these particular strains may represent an additional superinfection of the two goats with distinct SRLV strains circulating in the flock. Superinfection in humans with HIV-1 has been reported to occur when the superinfecting viruses do not share important B- and T-cell epitopes with the primary infecting virus, therefore escaping an efficient humoral and cellular immune defense (24, 33). The humoral immune response in goats is inefficient. Neutralizing antibodies are detected at low titers and only in a minority of persistently infected animals. This

FIG. 3. Multiple alignments of the nucleotide (A) and amino acid (B) sequences encompassing the C-terminal part of the surface (SU4-SU5) and N-terminal part of the TM portion of the *env* gene (bp positions 7482 to 8089 of CAEV-CO [accession number M33677]). The PBMC12-15 recombinant clone, the PBMC12-5 MVV-like clone, and the PBMC12-31 CAEV-like clone sequences are from goat 12, and the PBMC13-3 and the PBMC13-12 MVV-like clones and the PBMC13-24 CAEV-like clone are from goat 13. Dashes represent the gaps introduced to maximize the alignment, whereas points indicate sequence identity. The variable domains (SU4 and SU5) are delimited by horizontal arrows. Arrowheads define the cleavage site between the SU and the TM proteins. The sequences involved in the recombination event between CAEV and MVV are shaded in light gray for CAEV-like sequences and in dark gray for MVV-like sequences. A vertical arrow indicates the breakpoint of recombination. The PCR primers specific for the recombinant virus (the *recF* primer and the *recR* primer) are shaded in black.

may well favor super- or coinfections with heterologous viruses resisting this poor neutralizing response. The level and cross-reactivity of neutralizing antibody necessary to protect an individual from superinfection require further characterization.

Additionally, we confirmed that the HMA is a suitable technique to rapidly detect animals infected with diverse viruses (10). Detecting mixed infection relies upon adequate amplification of both viral species. It is critical that primers designed to detect mixed infection bind optimally to all genotypes so that the efficiency of PCR amplification of each coinfecting virus is the same. The sequence divergence of PCR products amplified from two coinfecting viruses is a key factor when using the HMA, and the SRLV infections detected by the HMA should not be confused with the presence of quasiespecies. The divergence between subtypes (>15%) and groups (>30%) is much greater than that seen between quasiespecies (<5%). The comparison between the sequencing data and the HMA results confirmed that our primers met these criteria. The reproducibility of the HMA pattern obtained from a given animal at different time points and from different sources such as colostrum, blood cells, or even coculture material provided strong evidence that these results cannot be explained by PCR contamination.

Finally, based on the results of a SimPlot analysis, we hypothesized that the peculiar sequence of clone PBMC12-15 may have resulted from a rare *in vivo* recombination event between the MVV- and CAEV-like viruses infecting goat 12. By using specific primers, we proved that this clone was not the product of an *in vitro* artifact but existed in different cell compartments, such as the blood and colostrum of goat 12 (data not shown). Additionally, the detection of both proviral DNA and viral RNA in GSM cell cocultures and of viral RNA in their supernatant demonstrates that this virus is capable of replicating independently or with the help of one or both of the parent viruses. Andr sd ttir (2) has shown that recombination is a frequent event in the envelope gene of MVV strain 1514 *in vitro* and *in vivo* and suggested that recombination may contribute to the high incidence of antigenic variation in MVV. The existence of SRLV carrying chimerical Env may have dramatic effects on the species specificity of these viruses and their capacity to cross the species barriers. Indeed, the absence of a functional CAEV receptor is the principal barrier protecting human cells from CAEV infection *in vitro*, the genome of which replicates without restrictions in transfected human cells (20). The apparent absence of recombinant viruses in the second goat analyzed may indicate that recombination events *in vivo* are rare or do not always involve the *env* gene but might occur in other regions of the genome that we aim to explore in future analyses.

In conclusion, we have demonstrated a dual infection with CAEV- and MVV-like viruses in two goats living under field conditions and presented strong evidence that these viruses can recombine *in vivo*. This illustrates a further mechanism used by SRLVs to evolve and increase their diversity and should support the continuation or initiation of eradication efforts in goat and sheep populations (25).

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REFERENCES

- Andr sd ttir, V., R. Skraban, S. Matthiasd ttir, R. Lutley, G. Agnarsd ttir, and H. Thorsteinsd ttir. 2002. Selection of antigenic variants in maedi-visna virus infection. *J. Gen. Virol.* **83**:2543–2551.
- Andr sd ttir, V. 2003. Evidence for recombination in the envelope gene of maedi-visna virus. *Virus Genes* **27**:5–9.
- Artenstein, A., T. VanCott, J. Mascola, J. Carr, P. Hegerich, J. Gaywee, E. Sanders-Buell, M. Robb, D. Dayhoff, S. Thitvichianlert, S. Nitayaphan, J. McNeil, D. Birx, R. Michael, D. Burke, and F. McCutchan. 1995. Dual infection with human immunodeficiency virus type 1 of distinct envelope subtypes in humans. *J. Infect. Dis.* **171**:805–810.
- Banks, K. L., D. S. Adams, T. C. McGuire, and J. Carlson. 1983. Experimental infection of sheep by caprine arthritis-encephalitis virus and goats by progressive pneumonia virus. *Am. J. Vet. Res.* **44**:2307–2311.
- Bertoni, G., C. Hertig, M. L. Zahno, H. R. Vogt, S. Dufour, P. Cordano, E. Peterhans, W. P. Cheevers, P. Sonigo, and G. Pancino. 2000. B-cell epitopes of the envelope glycoprotein of caprine arthritis-encephalitis virus and antibody response in infected goats. *J. Gen. Virol.* **81**:2929–2940.
- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513–1523.
- Butto, S., C. Argentini, A. M. Mazzella, M. P. Iannotti, P. Leone, A. Nicolosi, and G. Rezza. 1997. Dual infection with different strains of the same HIV-1 subtype. *AIDS* **11**:694–696.
- Delwart, E. L., E. G. Shpaer, J. Louwagie, F. E. McCutchan, M. Grez, H. Rubsamen-Waigmann, and J. I. Mullins. 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 *env* genes. *Science* **262**:1257–1261.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**:783–791.
- Germain, K., and S. Valas. 2006. Distribution and heterogeneity of small ruminant lentivirus envelope subtypes in naturally infected French sheep. *Virus Res.* **120**:156–162.
- Grez, M., U. Dietrich, P. Balfe, H. von Briesen, J. K. Maniar, G. Mahambre, E. L. Delwart, J. I. Mullins, and H. Rubsamen-Waigmann. 1994. Genetic analysis of human immunodeficiency virus type 1 and 2 (HIV-1 and HIV-2) mixed infections in India reveals a recent spread of HIV-1 and HIV-2 from a single ancestor for each of these viruses. *J. Virol.* **68**:2161–2168.
- Hotzel, L., N. Kumpula-McWhirter, and W. P. Cheevers. 2002. Rapid evolution of two discrete regions of the caprine arthritis-encephalitis virus envelope surface glycoprotein during persistent infection. *Virus Res.* **84**:17–25.
- Jolly, P. E., and O. Narayan. 1989. Evidence for interference, coinfections, and intertypic virus enhancement of infection by ovine-caprine lentiviruses. *J. Virol.* **63**:4682–4688.
- Kampinga, G., A. Simonon, P. van de Perre, E. Karita, P. Msellati, and J. Goudsmit. 1997. Primary infections with HIV-1 of women and their offspring in Rwanda: findings of heterogeneity at seroconversion, coinfection, and recombinants of HIV-1 subtypes A and C. *Virology* **227**:63–76.
- Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**:1244–1245.
- Leroux, C., J. Chastang, T. Greenland, and J. F. Mornex. 1997. Genomic heterogeneity of small ruminant lentiviruses: existence of heterogeneous populations in sheep and of the same lentiviral genotypes in sheep and goats. *Arch. Virol.* **142**:1125–1137.
- Liu, S.-L., A. G. Rodrigo, R. Shankarappa, G. H. Learn, L. Hsu, O. Davidov, L. P. Zhao, and J. I. Mullins. 1996. HIV quasiespecies and resampling. *Science* **273**:415–416.
- Meyerhans, A., J. P. Vartanian, and S. Wain-Hobson. 1990. DNA recombination during PCR. *Nucleic Acids Res.* **18**:1687–1691.
- Mordasini, F., H.-R. Vogt, M.-L. Zahno, A. Maeschli, C. Nenci, R. Zanoni, E. Peterhans, and G. Bertoni. 2006. Analysis of the antibody response to an immunodominant epitope of the envelope glycoprotein of a lentivirus and its diagnostic potential. *J. Clin. Microbiol.* **44**:981–991.
- Mselli-Lakhal, L., C. Favier, K. Leung, F. Guiguen, D. Grezel, P. Miossec, J. F. Mornex, O. Narayan, G. Querat, and Y. Chebloune. 2000. Lack of functional receptors is the only barrier that prevents caprine arthritis-encephalitis virus from infecting human cells. *J. Virol.* **74**:8343–8348.
- Narayan, O., J. E. Clements, J. D. Strandberg, L. C. Cork, and D. E. Griffin. 1980. Biological characterization of the virus causing leukoencephalitis and arthritis in goats. *J. Gen. Virol.* **50**:69–79.
- Pasick, J. 1998. Maedi-visna virus and caprine arthritis-encephalitis virus: distinct species or quasiespecies and its implications for laboratory diagnosis. *Can. J. Vet. Res.* **62**:241–244.
- Pepin, M., C. Vitu, P. Russo, J. F. Mornex, and E. Peterhans. 1998. Maedi-visna virus infection in sheep: a review. *Vet. Res.* **29**:341–367.

24. Pernas, M., C. Casado, R. Fuentes, M. J. Perez-Elias, and C. Lopez-Galindez. 2006. Dual superinfection and recombination within HIV-1 subtype B 12 years after primoinfection. *J. Acquir. Immune. Defic. Syndr.* **42**:12–18.
25. Peterhans, E., T. Greenland, J. Badiola, G. Harkiss, G. Bertoni, B. Amorena, M. Eliazewicz, R. A. Juste, R. Krassnig, J. P. Lafont, P. Lenihan, G. Petursson, G. Pritchard, J. Thorley, C. Vitu, J. F. Mornex, and M. Pepin. 2004. Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. *Vet. Res.* **35**:257–274.
26. Pisoni, G., A. Quasso, and P. Moroni. 2005. Phylogenetic analysis of small-ruminant lentivirus subtype B1 in mixed flocks: evidence for natural transmission from goats to sheep. *Virology* **339**:147–152.
27. Quérat, G., V. Barban, N. Sauze, P. Filippi, R. Vigne, P. Russo, and C. Vitu. 1984. Highly lytic and persistent lentiviruses naturally present in sheep with progressive pneumonia are genetically distinct. *J. Virol.* **52**:672–679.
28. Ravazzolo, A. P., D. Reischak, E. Peterhans, and R. Zanoni. 2001. Phylogenetic analysis of small ruminant lentiviruses from Southern Brazil. *Virus Res.* **79**:117–123.
29. Rolland, M., J. Mooney, S. Valas, G. Perrin, and R. Z. Mamoun. 2002. Characterization of an Irish caprine lentivirus strain: SRLV phylogeny revisited. *Virus Res.* **85**:29–39.
30. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
31. Shah, C., J. Böni, J. B. Huder, H.-R. Vogt, J. Mühlherr, R. Zanoni, R. Miserez, H. Lutz, and J. Schüpbach. 2004. Phylogenetic analysis and reclassification of caprine and ovine lentiviruses based on 104 new isolates: evidence for regular sheep-to-goat transmission and worldwide propagation through livestock trade. *Virology* **319**:12–26.
32. Shah, C., J. B. Huder, J. Böni, M. Schönmann, J. Mühlherr, H. Lutz, and J. Schüpbach. 2004. Direct evidence for natural transmission of small-ruminant lentiviruses of subtype A4 from goats to sheep and vice versa. *J. Virol.* **78**:7518–7522.
33. Smith, D. M., M. C. Strain, S. D. W. Frost, S. K. Pillai, J. K. Wong, T. Wrin, Y. Liu, C. J. Petropoulos, E. S. Daar, S. J. Little, and D. D. Richman. 2006. Lack of neutralizing antibody response to HIV-1 predisposes to superinfection. *Virology* **355**:1–5.
34. Tamura, K., and M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10**:512–526.
35. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
36. Thomson, M. M., and R. Nájera. 2005. Molecular epidemiology of HIV-1 variants in the global AIDS pandemic: an update. *AIDS Rev.* **7**:210–224.
37. Valas, S., C. Benoit, C. Baudry, G. Perrin, and R. Z. Mamoun. 2000. Variability and immunogenicity of caprine arthritis-encephalitis virus surface glycoprotein. *J. Virol.* **74**:6178–6185.
38. Zanoni, R. G. 1998. Phylogenetic analysis of small ruminant lentiviruses. *J. Gen. Virol.* **79**:1951–1961.
39. Zhu, T., N. Wang, A. Carr, S. Wolinsky, and D. D. Ho. 1995. Evidence for coinfection by multiple strains of human immunodeficiency virus type 1 subtype B in an acute seroconverter. *J. Virol.* **69**:1324–1327.